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### (54) Method for preparing an antitumor dextran

Verfahren zur Herstellung von Antitumordextran

Méthode de production de dextrane antitumoral

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## Description

BACKGROUND OF THE INVENTION5 Field of the Invention

This invention relates to microorganism capable of producing a dextran which has biological activities such as an excellent antitumor activity ; a method for producing said dextran using said microorganism or dextran synthetase prepared by said microorganism.

10 Description of the Prior Art

Methods for preparing antitumor polysaccharides using strains belonging to the genus Lactobacillus have already been disclosed in Japanese Patent Application Laid-Open Nos. 1-281073, 1-277484, 2-4714. However, these are crude products such as cells, capsular polysaccharide or culture broth.

The prior art methods described above provide species of microorganisms which belong to the genus Lactobacillus but are incapable of producing dextran. The present inventors have found that the dextrans produced by Lactobacillus confusus which is a species capable of producing a dextran have an antitumor activity. As a result of extensive investigations, a method for production thereof has been established and the present invention has thus come to be accomplished.

GB-A-2090846 discloses a high molecular weight polysaccharide MPS-80 which is obtained by culturing high molecular weight polysaccharide MPS-80- producing bacteria belonging to the genera Lactobacillus or Streptococcus. The new MPS-80 has an antitumor effect.

Patent abstracts of Japan, vol. 12, No. 283, page 50, C 518 corresponding to JP-A-63-61002 discloses a polysaccharide substance having antitumor activity which is obtained by culturing a microbial strain belonging to Lactobacillus genus.

SUMMARY OF THE INVENTION

The present invention relates to microorganism belonging to Lactobacillus confusus and capable of producing a dextran having the properties described below; a method for preparing said dextran using said microorganism or dextran synthetase produced by said microorganism.

- (1) Form: said dextran is a white powder having no taste and no smell;
- (2) Solubility: said dextran is soluble in water, formamide and dimethylsulfoxide but insoluble in alcohols, acetone, hexane, chloroform and carbon tetrachloride;
- (3) pH of Aquarious Solution: the aquarious solution is neutral or weakly acidic;
- (4) Constituent Saccharide: said dextran is composed solely of glucose;
- (5) Elementary analysis values: said dextran comprises 43 to 45% of C and 6.0 to 6.3% of H;
- (6) Structure: said dextran is an  $\alpha$ -glucan composed mainly of linear  $\alpha$ -1,6 glucoside linkages;
- (7) Proteins: said dextran contains no protein by the Lowry's method;
- (8) Molecular weight: said dextran does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
- (9) Color Reaction: said dextran is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (10) Melting Point: said dextran has no definite melting point;
- (11) Ultraviolet Absorption Spectrum: said dextran has no characteristic absorption as shown in Fig. 1;
- (12) Infrared Absorption Spectrum: said dextran shows a characteristic absorption of  $\alpha$ -glucan as shown in Fig. 2;
- (13)  $^{13}\text{C}$ -NMR Spectrum: said dextran shows characteristic spectrum of  $\alpha$ -1,6-glucan as shown in Fig. 3;
- (14) Said dextran possesses antitumor activities.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is UV absorption spectrum of the antitumor dextran obtained in the present invention. Fig. 2 is IR absorption spectrum of the same substance. Fig. 3 is  $^{13}\text{C}$ -NMR spectrum of the same substance.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In the present invention, the antitumor dextrans described above may be obtained by culturing a microorganism belonging to Lactobacillus confusus and capable of producing a dextran having the properties described above, accumulating a dextran in the culture broth and isolating the dextran. Furthermore, the antitumor dextran may also be obtained by acting dextran synthetase produced by the microorganism on sucrose and isolating the produced antitumor dextran.

As the microorganism capable of producing an antitumor dextran, there are Lactobacillus confusus 40-1 strain, 40-3 strain, 77-1 strain, 78-1 strain and 80-1 strain. Taxonomical properties of these strains are as follows.

Table I

Strain No.	40-1	40-3	77-1	78-1	80-1
Gram staining	+	+	+	+	+
Morphological characteristic	0.4 x 1-1.4 Short rod Single, pair Rarely chain	0.4 x 0.7-1.0 Short rod Single, pair Rarely chain	0.4 x 0.7-1.6 Short rod Single, pair Chain	0.4 x 0.7-2.0 Short rod Single, pair Rarely chain	0.4 x 0.7-1.4 Short rod Single, pair Chain
Catalase reaction	-	-	-	-	-
Oxidase reaction	-	-	-	-	-
Free oxygen demand	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic	Facultative anaerobic
Lactic acid fermentation	Hetero D,L	Hetero D,L	Hetero D,L	Hetero D,L	Hetero D,L
Decomposition of arginine	+	+	+	+	+
Final pH in a glucose medium	4.7	4.7	4.9	4.7	4.4

Table I (cont'd)

Strain No.	40-1	40-3	77-1	78-1	80-1
Decomposition of esclin	+	+	+	+	±
Dextran production	+	+	+	+	+
Growth in the presence of sodium chloride					
3.0% NaCl	+	+	+	+	+
6.0% NaCl	-	-	-	-	-
Production of acids from carbohydrate					
arabinose	+	+	+	+	±
glucose	+	+	+	+	+
fructose	+	+	+	+	+
lactose	+	+	+	+	+
mannose	+	+	+	+	+
trehalose	+	+	+	+	+

Summarizing the foregoing, the strains have the following properties. That is, 1: The microorganisms are positive in gram staining and facultative anaerobic. 2: Shape is short rod. 3: Acid production from trehalose is negative. 4: Lactic acid fermentation is hetero type and lactic acid of both D- and L-forms are produced. 5: Decomposition of arginine is positive. 6: Dextran production is positive.

Therefore, according to Bergey's Manual of Systematic Bacteriology, Vol. 2 (1986) and Method in Microbiology, Vol. 16, 147-178 (1984), these strains were identified to be Lactobacillus confusus. The present inventors named these strains as Lactobacillus confusus 40-1 strain (FERM BP-2865), 40-3 strain (FERM BP-2866), 77-1 strain (FERM BP-2867), 78-1 strain (FERM BP-2868) and 80-1 strain (FERM BP-2869), which were deposited at the Fermentation Research Institute, Agency of Industrial Science & Technology, Japan.

These *Lactobacillus confusus* 40-1 strain (FERM BP-2865); 40-3 strain (FERM BP-2866), 77-1 strain (FERM BP-2867), 78-1 strain (FERM BP-2868) and 80-1 strain (FERM BP-2869) are effective for producing the antitumor dextran in view of productivity.

Culture of the microorganism capable of producing the antitumor dextran is, in principle, carried out according to an ordinary microorganism-culture method. However, since *Lactobacillus confusus* is facultative anaerobic, and it demands no oxygen, it is advantageous to adopt stationary culture in liquid medium or gently agitating culture to unify the temperature. Where the antitumor dextran is produced directly in a culture broth, sucrose is essentially required as the carbon source for producing the antitumor dextran. Otherwise, a medium may be one containing nutrient sources the antitumor dextran-producing bacteria can utilize. Any of synthetic media, semi-synthetic media and natural media may be used.

As sucrose which is essentially required as a carbon source, any of crude to purified products of sucrose may be optionally used. For example, refined sugar, black sugar, molasses, blackstrap molasses, saccharose of reagent grade. A concentration of sucrose is 0.5 to 70%, preferably 5 to 50%. As nitrogen sources, there may be used meat extract, peptone, gluten meal, soybean powder, corn steep liquor, dry yeast extract, ammonium sulfate, urea. These nitrogen sources may be used, singly or mixture, and added to medium in a proportion of 0.5 to 5%, preferably 1 to 3%. In addition, phosphoric acid salts, sodium chloride, magnesium salts, cobalt salts, iron salts may be appropriately added to the medium, if necessary.

The incubation temperature is similar to the culture temperature used for ordinary mesophile bacteria, which is between 15 and 45°C, preferably between 20 and 30°C. The pH in culturing is 5 to 7. The incubation time is 5 to 96 hours, preferably 10 to 24 hours. By culturing under these conditions, the antitumor dextrans can be accumulated in the medium.

Where the antitumor dextrans are produced using dextran synthetase, sucrose is mandatorily required as the carbon source for accumulation of the antitumor dextran synthetase. A concentration of sucrose is preferably 0.1 to 5%. Other conditions for pH, temperature and incubation time are similar to those where the antitumor dextran is obtained directly in a culture broth.

In the thus obtained culture broth, the antitumor dextran synthetase is accumulated intracellularly and extracellularly. Therefore, when the enzyme is recovered from the cells, the broth is ultrasonically treated to disrupt the cells. Alternatively, the enzyme may be extracted from the cells with a surfactant, and the extract is then centrifuged, to remove insoluble matters. Further where the synthetase is recovered from the culture broth, the cells and insoluble matters may be removed by centrifugation, etc. The enzyme can be sufficiently used as a crude enzyme solution for production of the antitumor dextran as it is. If necessary, however, the enzyme may further be purified by dialysis, ultrafiltration, differential fractionation such as gel filtration, salting out with ammonium sulfate, treatments with ion exchange resins, singly or in combination.

By reacting the crude enzyme solution on sucrose, the antitumor dextran can be produced. The reaction is carried out at a temperature of 20 to 45°C, preferably 25 to 35°C for 5 to 50 hours at pH 5 to 7.

The produced antitumor dextran is usually contained in the medium or in the reaction mixture. Therefore, when the antitumor dextran is produced by fermentation, the antitumor dextran is purified by removing the cells or insoluble matters by means of centrifugation, filtration, then repeating precipitation procedures with polar organic solvents such as methanol, ethanol, propanol, acetone. Further by purifying dialysis, gel filtration, ultrafiltration, treatments with ion exchange resin, activated carbon, singly or in combination, the antitumor dextran of high purity can be obtained. By drying the product through spray drying, freeze drying, precipitation with polar organic solvents, the antitumor dextran can be obtained as a white powder.

Various properties of the anti-tumor dextran obtained by the foregoing procedures are given below.

- (1) Form: said dextran is a white powder having no taste and no smell;
- (2) Solubility: said dextran is soluble in water, formamide and dimethylsulfoxide but insoluble in alcohols, acetone, hexane, chloroform and carbon tetrachloride;
- (3) pH of Aquarous Solution: the aquarous solution is neutral or weakly acidic;
- (4) Constituent Saccharide: said dextran is composed solely of glucose;
- (5) Elementary analysis values: said dextran comprises 43.0 to 45% of C and 6.0 to 6.3% of H;
- (6) Structure: said dextran is an  $\alpha$ -glucan composed mainly of linear  $\alpha$ -1,6 glucoside linkages;
- (7) Proteins: said dextran contains no protein by the Lowry's method;
- (8) Molecular weight: said dextran does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
- (9) Color Reaction: said dextran is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (10) Melting Point: said dextran has no definite melting point;
- (11) Ultraviolet Absorption Spectrum: said dextran has no characteristic absorption as shown in Fig. 1;

(12) Infrared Absorption Spectrum: said dextran shows a characteristic absorption of  $\alpha$ -glucan as shown in Fig. 2.

(13)  $^{13}\text{C}$ -NMR Spectrum: said dextran shows characteristic spectrum of  $\alpha$ -1,6-glucan as shown in Fig. 3;

(14) Said dextran possesses antitumor activities.

The antitumor dextran obtained in the present invention can be degraded into low molecule through acid hydrolysis by gently heating in a diluted solution of sulfuric acid, hydrochloric acid, formic acid, enzymatic decomposition with a dextran decomposition enzyme, ultrasonication. The antitumor dextran degraded into low molecule can be fractionated by gel filtration, ultrafiltration, to render the molecular weight uniform. It is revealed that among the antitumor dextrans degraded into low molecular weight, dextrans having a molecular weight of 10,000 daltons or more has biological activities such as an antitumor activity.

Hereafter a method for assaying the antitumor activity and its results in experiments are given below when there are used the antitumor dextran obtained in Example 1 later described and low molecular products L1 (molecular weight of 1,000,000 or more), L2 (molecular weight of 100,000 to 1,000,000) and L3 (molecular weight of 10,000 to 100,000) of the antitumor dextran obtained in Example 4.

(a) Effect of the antitumor dextrans on syngeneic tumor, Meth-A by intraperitoneal administration

Meth-A tumor cells which had been subcultured in the peritoneal cavities of the same syngeneic mouse was intraperitoneally transplanted at a rate of  $1 \times 10^5$  cells/mouse to BALB/c-CRJ female mice of 6-week-old having a body weight of 20 g in average. The mice were divided into 4 groups: control group of 20 mice (1 group) and test groups of 10 mice (3 groups). For consecutive 5 days from the next day when the tumor cells were transplanted, the test groups were intraperitoneally administered 0.1 ml each of the solution of the antitumor dextran dissolved in saline at doses of 10, 30 and 100 mg/kg and the control group was similarly given by only saline. Subsequently, the number of survival days was observed and an increased life span was calculated by the following equation.

Increased life span (%) =

$$\frac{\text{Average survival days in the test groups}}{\text{Average survival days in the control group}} \times 100$$

(b) Effect of the antitumor dextran on syngeneic tumor Meth-A by oral administration

Meth-A tumor cells which had been subcultured in the peritoneal cavities of the same syngeneic mouse for a week was subcutaneously transplanted at a rate of  $6 \times 10^4$  to BALB/c-CRJ female mice of 6 weeks age having a body weight of 20 g in average. The mice were divided into 4 groups: control group of 20 mice (1 group) and test groups of 10 mice (3 groups). For consecutive 10 days from the next day when the tumor cells were transplanted, the test groups were administered orally using an oral sonde by 0.2 ml each of a saline solution of the antitumor dextran at doses of 10, 30 and 100 mg/kg and the control group was similarly given by only physiological saline. 35 Days after the tumor cells were transplanted, each mouse was killed and the propagated tumor was cut out and the weight was measured. The inhibition ratio was calculated based on the following equation.

Inhibition ratio (%) =

$$\left( 1 - \frac{\text{Average weight of tumor in test groups}}{\text{Average weight of tumor in control group}} \right) \times 100$$

The effects of the antitumor dextran assayed by the methods (a) and (b) described above are shown in Table II.

Table II

Dose	(a) Intraperitoneal administration		(b) Oral administration	
	Average number of survival day (day)	Increased life span (%)	Average weight of tumor (g)	Inhibition ratio (%)
Control (physiological saline)	18.7	-	12.10	-
Anti-tumor dextran:				
10 mg/kg	31.0	166	7.01	42
30 mg/kg	>42.0	>225	5.03	58
100 mg/kg	32.2	172	5.54	54

From the above table, it is revealed that the antitumor dextran possesses a potent antitumor activity with the optimum

dose of about 30 mg/kg, both by intraperitoneal administration and by oral administration.

Next, with respect to L1, L2 and L3 which are low molecular products of the antitumor dextran, the experiments similar to (a) and (b) described above were carried out in a dose of 30 mg/kg. The results are shown in Table III.

Table III

Dose	(a) Intraperitoneal administration		(b) Oral administration	
	Average number of survival day (day)	Increased life span (%)	Average weight of tumor (g)	Inhibition ratio (%)
Control (physiological saline)	18.7	-	12.10	-
L1 (30 mg/kg)	>42.0	>225	5.13	58
L2 (30 mg/kg)	>42.0	>225	5.72	53
L3 (30 mg/kg)	>42.0	>225	5.90	51

From the above table, it is revealed that the antitumor dextran degraded into low molecule by partial hydrolysis also retained the antitumor activity almost comparable to that prior to hydrolysis.

It is also confirmed that the antitumor dextran and its hydrolysate exhibit an excellent antitumor activity against syngeneic tumor Lewis lung tumor, melanoma B-16, allogeneic tumor sarcoma 180, Ehrlich tumor, by intraperitoneal administration or oral administration in a dose range of 10 to 100 mg/kg.

Next, acute toxicity of the antitumor dextran and its low molecular products L1, L2 and L3 will be described. Using ten 5-week-old male SD-CRJ rats having a body weight of 120 to 150 g as one group, the antitumor dextran, L1, L2 and L3 were orally administered to rats at a dose of 15 g/kg, which was the physical administration limit. During observation, no death of rats was noted and body weight increase was the same as that in the control group. In addition, no abnormality was noted at all in appearance and necropsy. Accordingly, it is considered that LD<sub>50</sub> would be larger than 15 g/kg and there would be no acute toxicity.

On the other hand, when the antitumor dextran was intravenously administered, LD<sub>50</sub> 280 mg/kg. And in case of L1, L2 and L3, their toxicity decreased with decrease of their molecular weight. In the case of L3, LD<sub>50</sub> was larger than 5 g/kg, and no toxicity was noted. Accordingly, the low molecular antitumor dextran has very advantageous properties when (it is) used as an injection drug.

For the practical production of medicines, the antitumor dextran or L1, L2 and L3 are used to produce medicines such as liquids, pills, tablets, powders, granules, suppositories, singly or in combination with a variety of excipients (water, physiological saline, polyethylene glycol, glycerol, gelatin, starch, dextrin, cellulose, lactose, mannitol).

In addition, the antitumor dextran may also be used as beverages or foodstuffs in functional foods for purposes of prevention of diseases or health care, or as food additives, since the antitumor dextran has a variety of biological activities useful for maintaining health through oral administration and is readily processed because of tasteless and odorless nature.

[Examples]

Next, the present invention is described in more detail by referring to the examples.

#### Example 1

(Culture)

Cells obtained from stab culture of *Lactobacillus confusus* 40-1 strain (FERM BP-2865) were inoculated into 5 ml of medium (2.0% of sucrose, 0.5% of yeast extract, 2.0% of K<sub>2</sub>HPO<sub>4</sub>, pH 7.4) in a test tube of 15 mm in diameter, the stationary culture was carried out at 26°C for 24 hours. Then, 5 ml of the medium was inoculated on 400 ml of medium having the same composition in an 500 ml Erlenmeyer's flask, and the stationary culture was carried out at 26°C for 24 hours.

The obtained culture broth, 400 ml, was inoculated on 20 liters of SMI medium (10% of sucrose, 0.05% of yeast extract, 0.5% of K<sub>2</sub>HPO<sub>4</sub>, 0.1% of sodium chloride, pH 7.4) in a 30 liters jar fermenter. While gently agitating (0.2 v/v/m, 10 rpm) at 26°C for 15 hours in a nitrogen flow, cultivation was carried out.



## (Purification)

After adjusting pH of culture broth to 7, the medium was heated at 100°C for the purpose of sterilization. Then, the cells and insoluble matters were removed with a continuous centrifuging machine to give 18.6 liters of the culture supernatant.

Methanol was gradually added to the culture supernatant with agitating, in a final concentration of 45% (v/v) and the mixture was allowed to stand. After the supernatant was removed by decantation, the resulting precipitates were washed with 60% (v/v) methanol. The precipitates were dissolved in 20 liters of deionized water again. The precipitation with methanol and washing with 60% (v/v) methanol were performed 3 times in total. Then by spray drying 270 g of the antitumor dextran was obtained as a white powder.

## Example 2

Culture and purification were performed in a same manner as in Example 1 except that blackstrap molasses was added to SMI medium in a concentration of 10% instead of sucrose. The resulting precipitates were dissolved in 3 liters of deionized water. Then by freeze drying, 251 g of the antitumor dextran was obtained as a white powder.

## Example 3

Methanol was gradually added with agitating to 100 ml of the culture supernatant obtained in Example 1 in a final concentration of 45% (v/v). After the obtained glutinous precipitates were washed with 60% (v/v) methanol, the precipitates were dissolved in 100 ml of deionized water again. The solution was passed through DEAE TOYOPEARL 650 M $\phi$  which had been equilibrated with deionized water. The non-absorbed fraction was collected and concentrated. After desalting and then freeze drying, 1.1 g of the antitumor dextran was obtained.

## Example 4

After 5.0 g of the antitumor dextran powder obtained in Example 1 was dissolved in 500 ml of 2% sulfuric acid, the solution was partially hydrolyzed at 60°C for 4 hours. After neutralizing with barium carbonate, the precipitates were removed by centrifugation. Ultrafiltration was performed using membranes of 1,000,000, 100,000 and 10,000 for fractionation of molecular weights in this order to give 3 fractions of L1, L2 and L3, namely, L1 (molecular weight of 1,000,000 or more), L2 (molecular weight of 100,000 to 1,000,000) and L3 molecular weight of 10,000 to 100,000), respectively. By freeze drying each fraction, the low molecular antitumor dextran of L1: 1.6 g, L2: 1.2 g and L3: 0.9 g were obtained, respectively as a white powder.

## Example 5

The procedures were performed in a same manner as in Example 1 except that Lactobacillus confusus 40-3 strain (FERM BP-2866) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 263 g of the antitumor dextran was obtained as a white powder.

## Example 6

The procedures were performed in a same manner as in Example 1 except that Lactobacillus confusus 77-1 strain (FERM BP-2867) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 212 g of the antitumor dextran was obtained as a white powder.

## Example 7

The procedures were performed in a same manner as in Example 1 except that Lactobacillus confusus 78-1 strain (FERM BP-2868) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 250 g of the antitumor dextran was obtained as a white powder.

## Example 8

The procedures were performed in a same manner as in Example 1 except that Lactobacillus confusus 80-1 strain (FERM BP-2869) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 209 g of the anti-tumor dextran was obtained as a white powder.

## Example 9

Cells obtained from stab culture of Lactobacillus confusus 40-1 strain (FERM BP-2865) were inoculated on 5 ml of medium (2.0% of sucrose, 0.5% of yeast extract, 2.0% of  $K_2HPO_4$ , pH 7.4) in a test tube of 15 mm in diameter, and stationary culture was carried out at 26°C for 24 hours.

Then, 5 ml of the medium was inoculated on 400 ml of medium having the same composition in an 500 ml Erlenmeyer's flask, and stationary culture was carried out at 26°C for 17 hours. The obtained medium was centrifuged (10,000G, 15 minutes) to give 360 ml of the supernatant. The supernatant was concentrated by ultrafiltration membrane (Amicon®, hollow fiber, fractional molecular weight of 10,000) and then replaced by 5 mM acetate buffer (pH 5.5) to give 100 ml of the crude enzyme solution. After adding a small quantity of toluene, the crude enzyme solution was stored at 4°C.

After 20 ml of the obtained crude enzyme solution was mixed with 60 ml of 50 mM acetate buffer (pH 5.5) in which 8 g of sucrose had been dissolved, a small quantity of toluene was added to the mixture. The mixture was gently agitated at 30°C for 15 hours. Methanol was added to the resulting viscous solution in a final concentration of 40% (v/v) to precipitate the antitumor dextran.

After washing with 60% (v/v) methanol, the obtained precipitates were dissolved in 80 ml of deionized water again. The precipitation with methanol and washing with 60% (v/v) methanol were repeated. After dissolving in deionized water and freeze drying, 3.2 g of the antitumor dextran was obtained as a white powder.

## Example 10

After 10 g of sucrose was dissolved in 100 ml of the culture supernatant obtained in Example 9, a small quantity of toluene was added to the solution. While mildly agitating, the solution was kept at 30°C for 18 hours. 150 ml of methanol was added with stirring to the resulting viscous solution (final concentration of 43%) to give glutinous precipitates. After washing with 60% (v/v) methanol, the precipitates were dissolved in 100 ml of deionized water again. The precipitation with methanol and washing with 60% (v/v) methanol were repeated. After dissolving in deionized water and freeze drying the solution, 3.9 g of the antitumor dextran was obtained as a white powder.

## Example 11

Cells obtained from stab culture of Lactobacillus confusus 40-1 strain (FERM BP-2865) were inoculated on 5 ml of medium (2.0% of sucrose, 0.5% of yeast extract, 2.0% of  $K_2HPO_4$ , pH 7.4) in a test tube of 15 mm in diameter the stationary culture was carried out at 26°C for 24 hours. Then, 5 ml of the medium was inoculated on 400 ml of medium having the same composition charged in an 500 ml Erlenmeyer's flask, and stationary culture was carried out at 26°C for 17 hours.

After the cells in the culture broth (350 ml) was disrupted with a ultrasonic homogenizer, insoluble matters were removed by centrifugation to give 330 ml of the supernatant. The resulting supernatant was treated in a same manner to Example 9 to give 80 ml of the crude enzyme solution. After 20 ml of the obtained crude enzyme solution was mixed with 100 ml of 50 mM acetate buffer (pH 5.5) in which 12 g of sucrose had been dissolved, a small quantity of toluene was added to the solution. The mixture was mildly agitated at 30°C for 15 minutes. Methanol was added to the resulting viscous solution in a final concentration of 40% to precipitate the antitumor dextran. After washing with 60% (v/v) methanol, the precipitates were dissolved in 120 ml of deionized water again. The precipitation with methanol and washing with 60% (v/v) methanol were repeated. After dissolving in deionized water and freeze drying, 5.0 g of the antitumor dextran was obtained as a white powder.

## Example 12

The procedures were performed in a same manner as in Example 9 except that Lactobacillus confusus 40-3 strain (FERM BP-2866) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 2.7 g of the anti-tumor dextran was obtained as a white powder.

## Example 13

The procedures were performed in a same manner as in Example 9 except that Lactobacillus confusus 77-1 strain (FERM BP-2867) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 2.1 g of the antitumor dextran was obtained as a white powder.

## Example 14

The procedures were performed in a same manner as in Example 9 except that Lactobacillus confusus 78-1 strain (FERM BP-2868) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 2.4 g of the antitumor dextran was obtained as a white powder.

## Example 15

The procedures were performed in a same manner as in Example 9 except that Lactobacillus confusus 80-1 strain (FERM BP-2869) was used instead of Lactobacillus confusus 40-1 strain (FERM BP-2865). Thus, 3.4 g of the antitumor dextran was obtained as a white powder.

The antitumor dextran of the present invention which is a pure glucan, has biological activities such as an excellent antitumor activity and has no toxicity can be stably prepared by a simple and efficient method.

## Claims

## 1. A method for preparing an antitumor dextran which comprises

(a) culturing in the presence of sucrose as a source of carbon a microorganism belonging to Lactobacillus confusus, which has an ability of producing a dextran having the following properties:

- (1) Form: said dextran is a white powder having no taste and no smell;
- (2) Solubility: said dextran is soluble in water, formamide and dimethylsulfoxide but insoluble in alcohols, acetone, hexane, chloroform and carbon tetrachloride;
- (3) pH of Aquarious Solution: the aquarious solution is neutral or weakly acidic;
- (4) Constituent Saccharide: said dextran is composed solely of glucose;
- (5) Elementary analysis values: said dextran comprises 43 to 45% of C and 6.0 to 6.3% of H;
- (6) Structure: said dextran is an  $\alpha$ -glucan composed mainly of linear  $\alpha$ -1,6 glucoside linkages;
- (7) Proteins: said dextran contains no protein by the Lowry's method;
- (8) Molecular weight: said dextran does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltons;
- (9) Color Reaction: said dextran is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (10) Melting Point: said dextran has no definite melting point;
- (11) Ultraviolet Absorption Spectrum: said dextran has no characteristic absorption;
- (12) Infrared Absorption Spectrum: said dextran shows a characteristic absorption of  $\alpha$ -glucan;
- (13)  $^{13}\text{C}$ -NMR Spectrum: said dextran shows characteristic spectrum of  $\alpha$ -1,6-glucan;
- (14) Said dextran possesses antitumor activities.

(b) collecting said dextran from culture broth.

2. A method according to claim 1, wherein said microorganism capable of producing an antitumor dextran is one microorganism selected from the group consisting of Lactobacillus confusus 40-1 strain (FERM BP-2865), 40-3 strain (FERM BP-2866), 77-1 strain (FERM BP-2867), 78-1 strain (FERM BP-2868), 80-1 strain (FERM BP-2869) and mutants thereof.

## 3. A method for preparing an antitumor dextran which comprises

(a) contacting sucrose with an antitumor dextran synthetase derived from a culture broth of a microorganism belonging to Lactobacillus confusus, which has an ability of producing a dextran having the following properties:

- (1) Form: said dextran is a white powder having no taste and no smell;
- (2) Solubility: said dextran is soluble in water, formamide and dimethylsulfoxide but insoluble in alcohols, acetone, hexane, chloroform and carbon tetrachloride;
- (3) pH of Aquarious Solution: the aquarious solution is neutral or weakly acidic;
- (4) Constituent Saccharide: said dextran is composed solely of glucose;
- (5) Elementary analysis values: said dextran comprises 43 to 45% of C and 6.0 to 6.3% of H;

- (6) Structure: said dextran is an  $\alpha$ -glucan composed mainly of linear  $\alpha$ -1,6 glucoside linkages;
- (7) Proteins: said dextran contains no protein by the Lowry's method;
- (8) Molecular weight: said dextran does not permeate through a dialysis membrane and the molecular weight is presumed to be higher than 10,000 daltones;
- (9) Color Reaction: said dextran is positive to anthrone sulfuric acid reaction and phenol sulfuric acid reaction, but negative to biuret reaction, the Lowry-Folin reaction, the Elson-Morgan reaction and iodine reaction;
- (10) Melting Point: said dextran has no definite melting point;
- (11) Ultraviolet Absorption Spectrum: said dextran has no characteristic absorption;
- (12) Infrared Absorption Spectrum: said dextran shows a characteristic absorption of  $\alpha$ -glucan;
- (13)  $^{13}\text{C}$ -NMR Spectrum: said dextran shows characteristic spectrum of  $\alpha$ -1,6-glucan;
- (14) Said dextran possesses antitumor activities.

(b) collecting said dextran.

4. A method according to claim 3, wherein said microorganism capable of producing an antitumor dextran is one microorganism selected from the group consisting of *Lactobacillus confusus* 40-1 strain (FERM BP-2865), 40-3 strain (FERM BP-2866), 77-1 strain (FERM BP-2867), 78-1 strain (FERM BP-2868), 80-1 strain (FERM BP-2869) and mutants thereof.
5. *Lactobacillus confusus* 40-1 strain (FERM BP-2865), 40-3 strain (FERM BP-2866), 77-1 strain (FERM BP-2867), 78-1 strain (FERM BP-2868) and 80-1 strain (FERM BP-2869).

#### Patentansprüche

1. Verfahren zur Herstellung eines Antitumor-Dextrans, das umfaßt:

a) das Kultivieren in Gegenwart von Saccharose als Kohlenstoffquelle eines Mikroorganismus, der zu *Lactobacillus confusus* gehört, der die Fähigkeit hat, ein Dextran mit den folgenden Eigenschaften zu bilden:

- 1) Form: das genannte Dextran ist ein geschmack- und geruchloses weißes Pulver;
- 2) Löslichkeit: das genannte Dextran ist in Wasser, Formamid und Dimethylsulfoxid löslich, jedoch in Alkoholen, Aceton, Hexan, Chloroform und Tetrachlorkohlenstoff unlöslich;
- 3) pH-Wert einer wäßrigen Lösung: die wäßrige Lösung ist neutral oder schwach sauer;
- 4) Saccharid-Bestandteil: das genannte Dextran besteht nur aus Glucose;
- 5) Elementaranalysen-Werte: das genannte Dextran enthält 43 bis 45 % C und 6,0 bis 6,3 % H;
- 6) Struktur: das genannte Dextran ist ein  $\alpha$ -Glucan, das hauptsächlich aus linearen  $\alpha$ -1,6-Glucosid-Bindungen besteht;
- 7) Proteine: das genannte Dextran enthält kein Protein nach der Lowry-Methode;
- 8) Molekulargewicht: das genannte Dextran durchdringt nicht eine Dialysemembran und das Molekulargewicht beträgt, wie angenommen wird, mehr als 10 000 Dalton;
- 9) Farbreaktion: das genannte Dextran ist positiv gegenüber der Anthron-Schwefelsäure-Reaktion und der Phenol-Schwefelsäure-Reaktion, jedoch negativ gegenüber der Biuret-Reaktion, der Lowry-Folin-Reaktion, der Elson-Morgan-Reaktion und der Jod-Reaktion;
- 10) Schmelzpunkt: das genannte Dextran hat keinen definierten Schmelzpunkt;
- 11) Ultraviolett-Absorptionsspektrum: das genannte Dextran weist keine charakteristische Absorption auf;
- 12) Infrarot-Absorptionsspektrum: das genannte Dextran weist eine charakteristische Absorption von  $\alpha$ -Glucan auf;
- 13)  $^{13}\text{C}$ -NMR-Spektrum: das genannte Dextran weist ein charakteristisches Spektrum von  $\alpha$ -1,6-Glucan auf;
- 14) das genannte Dextran weist Antitumor-Aktivitäten auf, und

b) das Sammeln des Dextrans aus der Kulturbrühe.

2. Verfahren nach Anspruch 1, worin der genannte Mikroorganismus, der ein Antitumor-Dextran bilden kann, ein Mikroorganismus ist, der ausgewählt wird aus der Gruppe, die besteht aus *Lactobacillus confusus*, Stamm 40-1 (FERM BP-2865), Stamm 40-3 (FERM BP-2866), Stamm 77-1 (FERM BP-2867), Stamm 78-1 (FERM BP-2868), Stamm 80-1 (FERM BP-2869) und Mutanten davon.

## 3. Verfahren zur Herstellung eines Antitumor-Dextrans, das umfaßt:

a) das Inkontaktbringen von Saccharose mit einer Antitumor-Dextran-Synthetase, die stammt aus einer Kulturbrothe eines Mikroorganismus, der zu *Lactobacillus confusus* gehört, der die Fähigkeit hat, ein Dextran mit den folgenden Eigenschaften zu bilden:

- 1) Form: das genannte Dextran ist ein geschmack- und geruchloses weißes Pulver;
- 2) Löslichkeit: das genannte Dextran ist in Wasser, Formamid und Dimethylsulfoxid löslich, jedoch in Alkoholen, Aceton, Hexan, Chloroform und Tetrachlorkohlenstoff unlöslich;
- 3) pH-Wert einer wäßrigen Lösung: die wäßrige Lösung ist neutral oder schwach sauer;
- 4) Saccharid-Bestandteil: das genannte Dextran besteht nur aus Glucose;
- 5) Elementaranalysen-Werte: das genannte Dextran enthält 43 bis 45 % C und 6,0 bis 6,3 % H;
- 6) Struktur: das genannte Dextran ist ein  $\alpha$ -Glucan, das hauptsächlich aus linearen  $\alpha$ -1,6-Glucosid-Bindungen besteht;
- 7) Proteine: das genannte Dextran enthält kein Protein nach der Lowry-Methode;
- 8) Molekulargewicht: das genannte Dextran durchdringt nicht eine Dialysemembran und das Molekulargewicht beträgt, wie angenommen wird, mehr als 10 000 Dalton;
- 9) Farbreaktion: das genannte Dextran ist positiv gegenüber der Anthron-Schwefelsäure-Reaktion und der Phenol-Schwefelsäure-Reaktion, jedoch negativ gegenüber der Biuret-Reaktion, der Lowry-Folin-Reaktion, der Elson-Morgan-Reaktion und der Jod-Reaktion;
- 10) Schmelzpunkt: das genannte Dextran hat keinen definierten Schmelzpunkt;
- 11) Ultraviolett-Absorptionsspektrum: das genannte Dextran weist keine charakteristische Absorption auf;
- 12) Infrarot-Absorptionsspektrum: das genannte Dextran weist eine charakteristische Absorption von  $\alpha$ -Glucan auf;
- 13)  $^{13}\text{C}$ -NMR-Spektrum: das genannte Dextran weist ein charakteristisches Spektrum von  $\alpha$ -1,6-Glucan auf;
- 14) das genannte Dextran weist Antitumor-Aktivitäten auf, und

b) das Sammeln des genannten Dextrans.

4. Verfahren nach Anspruch 3, worin der Mikroorganismus, der ein Antitumor-Dextran bilden kann, ein Mikroorganismus ist, der ausgewählt wird aus der Gruppe, die besteht aus *Lactobacillus confusus*, Stamm 40-1 (FERM BP-2865), Stamm 40-3 (FERM BP-2866), Stamm 77-1 (FERM BP-2867), Stamm 78-1 (FERM BP-2868), Stamm 80-1 (FERM BP-2869) und Mutanten davon.
5. *Lactobacillus confusus*, Stamm 40-1 (FERM BP-2865), Stamm 40-3 (FERM BP-2866), Stamm 77-1 (FERM BP-2867), Stamm 78-1 (FERM BP-2868) und Stamm 80-1 (FERM BP-2869).

## 40 Revendications

## 1. Procédé de préparation d'un dextrane antitumoral qui comprend:

(a) la culture, en présence de saccharose en tant que source de carbone, d'un micro-organisme appartenant à l'espèce *Lactobacillus confusus*, qui est capable de produire un dextrane possédant les propriétés suivantes:

- (1) Forme: ledit dextrane est une poudre blanche inodore et sans saveur;
- (2) Solubilité: ledit dextrane est soluble dans l'eau, le formamide et le diméthylsulfoxyde mais insoluble dans les alcools, l'acétone, l'hexane, le chloroforme et le tétrachlorure de carbone;
- (3) pH dudit dextrane en solution aqueuse: la solution aqueuse est neutre ou faiblement acide;
- (4) Constituant saccharidique: ledit dextrane est exclusivement composé de glucose;
- (5) Analyse élémentaire: ledit dextrane comprend 43 à 45% de C et 6,0 à 6,3% de H;
- (6) Structure: ledit dextrane est un composé d' $\alpha$ -glycane essentiellement formé par des liaisons glycosidiques linéaires  $\alpha$ -1,6;
- (7) Protéines: ledit dextrane ne contient pas de protéines décelables par la méthode de Lowry;
- (8) Poids moléculaire: ledit dextrane ne traverse pas une membrane de dialyse et son poids moléculaire est supposé être supérieur à 10.000 daltons;
- (9) Réaction colorimétrique: ledit dextrane réagit positivement avec l'anthrone-acide sulfurique et le phé-

nol-acide sulfurique mais donne un résultat négatif à la réaction de biuret, la réaction de Lowry-Folin, la réaction de Elson-Morgan et la réaction à l'iode;

(10) Point de fusion: ledit dextrane n'a pas de point de fusion défini;

(11) Spectre d'absorption dans l'ultraviolet: ledit dextrane n'a pas d'absorption caractéristique;

(12) Spectre d'absorption dans l'infrarouge: ledit dextrane présente le spectre caractéristique de l' $\alpha$ -glycane;

(13) Spectre de RMN du  $^{13}\text{C}$ : ledit dextrane présente le spectre caractéristique de l' $\alpha$ -1,6-glycane;

(14) Ledit dextrane possède des activités antitumorales.

(b) la récupération dudit dextrane dans le milieu de culture.

2. Procédé selon la revendication 1, dans lequel ledit micro-organisme capable de produire un dextrane antitumoral est un micro-organisme choisi au sein du groupe formé par les souches 40-1 (FERM BP-2865), 40-3 (FERM BP-2866), 77-1 (FERM BP-2867), 78-1 (FERM BP-2868), 80-1 (FERM BP-2869) de Lactobacillus confusus et les formes mutantes de ces souches.

3. Procédé de préparation d'un dextrane antitumoral qui comprend:

(a) la mise en contact du saccharose avec une dextrane antitumoral synthétase dérivée du milieu de culture d'un micro-organisme appartenant à l'espèce Lactobacillus confusus, qui est capable de produire un dextrane possédant les propriétés suivantes:

(1) Forme: ledit dextrane est une poudre blanche inodore et sans saveur;

(2) Solubilité: ledit dextrane est soluble dans l'eau, le formamide et le diméthylsulfoxyde mais insoluble dans les alcools, l'acétone, l'hexane, le chloroforme et le tétrachlorure de carbone;

(3) pH dudit dextrane en solution aqueuse: la solution aqueuse est neutre ou faiblement acide;

(4) Constituant saccharidique: ledit dextrane est exclusivement composé de glucose;

(5) Analyse élémentaire: ledit dextrane comprend 43 à 45% de C et 6,0 à 6,3% de H;

(6) Structure: ledit dextrane est un composé d' $\alpha$ -glycane essentiellement formé par des liaisons glycosidiques linéaires  $\alpha$ -1,6;

(7) Protéines: ledit dextrane ne contient pas de protéines décelables par la méthode de Lowry;

(8) Poids moléculaire: ledit dextrane ne traverse pas une membrane de dialyse et son poids moléculaire est supposé être supérieur à 10.000 daltons;

(9) Réaction colorimétrique: ledit dextrane réagit positivement avec l'anthrone-acide sulfurique et le phénol-acide sulfurique mais donne un résultat négatif à la réaction de biuret, la réaction de Lowry-Folin, la réaction de Elson-Morgan et la réaction à l'iode;

(10) Point de fusion: ledit dextrane n'a pas de point de fusion défini;

(11) Spectre d'absorption dans l'ultraviolet: ledit dextrane n'a pas d'absorption caractéristique;

(12) Spectre d'absorption dans l'infrarouge: ledit dextrane présente le spectre caractéristique de l' $\alpha$ -glycane;

(13) Spectre de RMN du  $^{13}\text{C}$ : ledit dextrane présente le spectre caractéristique de l' $\alpha$ -1,6-glycane;

(14) Ledit dextrane possède des activités antitumorales.

(b) la récupération dudit dextrane dans le milieu de culture.

4. Procédé selon la revendication 3, où ledit micro-organisme capable de produire un dextrane antitumoral est un micro-organisme choisi au sein du groupe formé par les souches 40-1 (FERM BP-2865), 40-3 (FERM BP-2866), 77-1 (FERM BP-2867), 78-1 (FERM BP-2868), 80-1 (FERM BP-2869) de Lactobacillus confusus et les formes mutantes de ces souches.

5. Souches 40-1 (FERM BP-2865), 40-3 (FERM BP-2866), 77-1 (FERM BP-2867), 78-1 (FERM BP-2868), 80-1 (FERM BP-2869) de Lactobacillus confusus.

**FIG. 1**

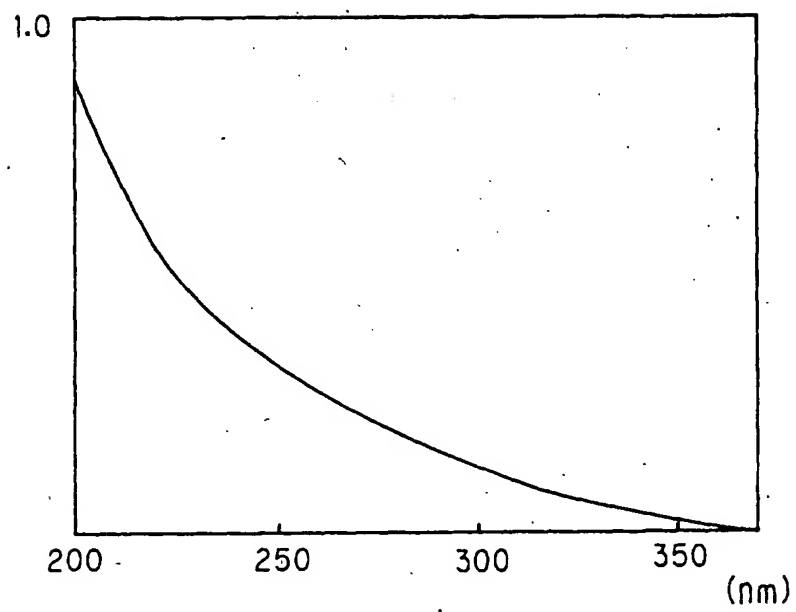
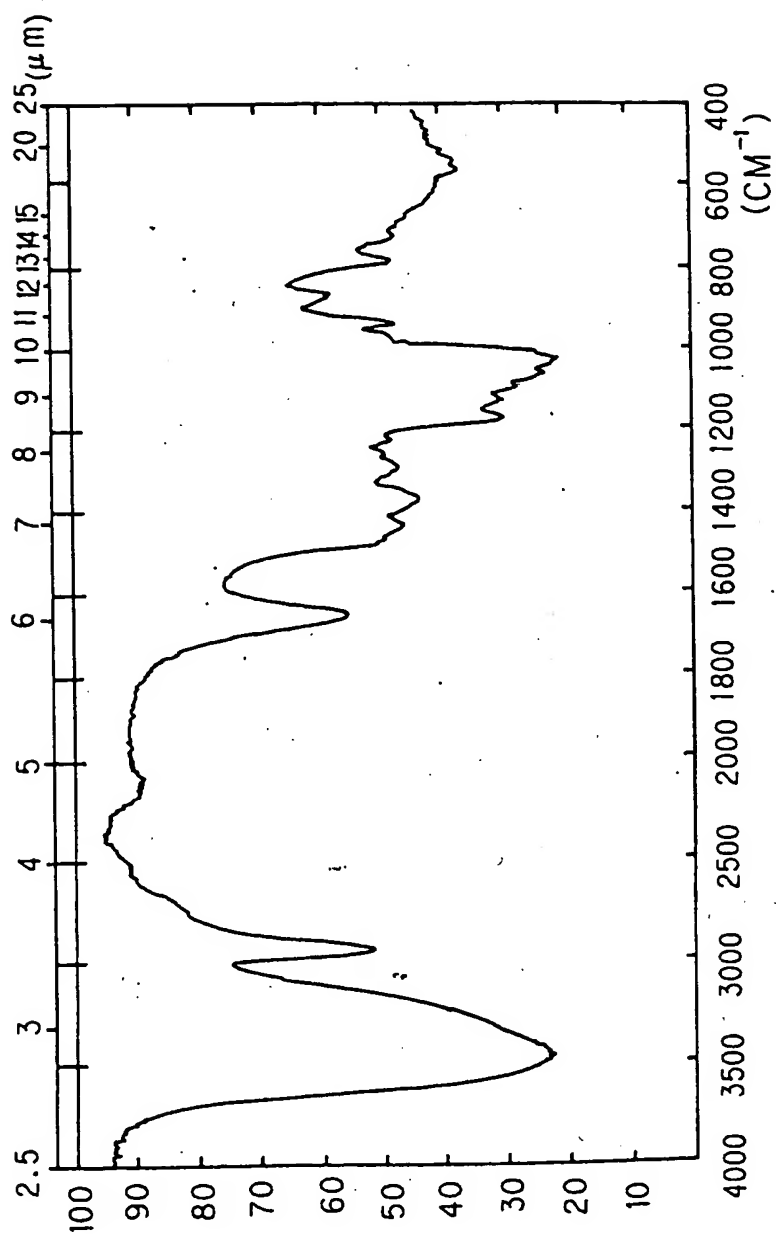


FIG. 2





*FIG. 3*

